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ORIGINAL ARTICLE

Role of Polymerase Chain Reaction (PCR) in the detection of antibiotic-resistant *Staphylococcus aureus*

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Abstract *Background:* *Staphylococcus aureus* is mainly acquired from hospital infections and demonstrated the ability of developing resistance to many antibiotics. Polymerase Chain Reaction (PCR) was used to identify antibiotic-resistant isolates. This study was conducted in Al-Mujtahed, Al-Mouwasat and the Children Hospitals in Damascus during the period between January and June in 2013.

Objectives: This study aimed to investigate *S. aureus* in some clinical samples by PCR and study the bacterial resistance to some antibiotics.

Materials and methods: DNA fragments were amplified from isolated DNA. PCR was used to amplify the sequences of 16S rRNA, gap gene and nuc gene depending on six specific primers. The PCR products were detected by agarose gel electrophoresis. The antibiotics susceptibility tests were conducted on all isolates using the Kirby-Bauer disk diffusion method on Mueller Hinton agar and Luria Bertani (LB) Agar.

Results: Eighty one isolates of *S. aureus* were collected from blood samples, urine samples and bronchial secretions. The results showed that the DNA fragments of 16S rRNA, gap gene and nuc gene were approximately equal to 479 bp, 933 bp and 270 bp, respectively and the results of antibiotics resistance for the 10 tested antibiotics were as following: Chloramphenicol (97.5%), Tetracycline (50.6%), Cefuroxime (37.0%), Oxacillin (33.3%), Levofloxacin (37.0), Erythromycin (35.8%), Ciprofloxacin (32.1%), Rifampicin (7.4%), Vancomycin (3.7%), Imipenem (0%).

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Conclusion: This study showed that the PCR is a specific and effective method for classifying and identifying isolates of *S. aureus*, which demonstrated increasing resistance against many antibiotics.

1. Introduction

S. aureus is considered an important factor of infection either acquired from community or hospital infections. It may also leads to serious complications, such as pneumonia, septicemia, arthritis and osteomyelitis [1]. During the previous five decades, *S. aureus* was cloned as methicillin-resistant (MRSA) resulting in medical and public health problems worldwide [2]. It is responsible for diseases caused by exotoxin production (toxic shock and staphylococcal scalded-skin syndromes). It was isolated from direct invasion, such as urinary tract infection (UTI) and it is isolated from Intensive Care Units [3,4]. Furthermore, *S. aureus* is responsible for many suppurative lesions and demonstrated the ability of developing resistance to many antibiotics especially among the patients residing in hospitals [5]. Methicillin-resistant *S. aureus* is conferred by the staphylococcal cassette chromosome mec (SCCmec) elements, which carry the mecA gene encoding a penicillin-binding protein homologue (PBP2a), with reduced affinity for β-lactams antibiotics [6]. Some reports revealed that *S. aureus* evolves resistance to many classes of antibiotics [7]. The emergence of antibiotic-resistance *S. aureus* strains resulted in significant treatment difficulties which imposed burden on health care systems and simultaneously intensifying the need for new antibiotics [8]. Recently many PCR based molecular methods were developed as an alternative ways for accurate identification [9]. Amplification of 16S rRNA gene sequences (~479 bp) is the most commonly used method for identifying and classifying bacteria, including staphylococci [10,11]. The reports indicated that the sequences of the gap gene (~933 bp) are composed of 12 species relevant for human. The gap gene encodes a 42-kDa transferring-binding protein (Tpn) located within the cell wall of the *Staphylococci* [12]. The gap gene also encodes cell wall enzyme (Glyceraldehyde-3-Phosphate Dehydrogenase) and it has been commonly considered a constitutive housekeeping gene [12,13]. Identification of *S. aureus* using PCR amplification of the nuc gene (~270 bp) is considered as a gold standard method [14]. A nuc gene encodes the thermonuclease enzyme where experiments suggested a major role for nuc1 in terms of the thermonuclease activity in *S. aureus* [15]. The nuc gene in *S. aureus* encodes the thermonuclease enzyme and amplification of nuc gene is potential for rapid diagnosis of *S. aureus* infection [16].

2. Materials and methods

2.1. Bacterial identification

In this study 81 isolates of *S. aureus* were investigated from different sources, such as blood samples, urine samples, bronchial secretions. This study was conducted at Al-Mujtahed, Al-Mouwasat and The Children Hospitals in Damascus. The isolates were cultured on Luria Bertani Agar (LB Agar) and Mannitol Salt Agar (MSA). Gram stain was performed for all isolates which were then examined under the microscope.

2.2. DNA isolation

S. aureus was cultured on 5 ml Luria Bertani broth and incubated at 37 °C for 24 h. Afterwards, 1.5 ml of the culture was centrifuged for 2 min at a velocity of (14,000 rpm). Pellet was re-suspended in 567 µl Tris–EDTA buffer by repeated pipetting or vortexing. 3 µl of 10% SDS and 3 µl of 20 mg/ml proteinase K were added, mixed and incubated for 1 h at 37 °C with shaking (1000 rpm) in a thermomixer machine. After that, 80 µl of Cetyl trimethyl ammonium bromide (CTAB)/NaCl solution was added, mixed and incubated for 10 min at 65 °C with shaking (1000 rpm). A volume of chloroform/isoamyl-alcohol equal to (~700 µl) was added, mixed, centrifuged for 5 min at speeding (14,000 rpm) and the supernatant was transferred to fresh tube. Afterwards, a volume of phenol/chloroform/isoamyl-alcohol equal to (~600 µl) was mixed for 5 min, centrifuged and the supernatant transferred to a fresh tube, then 0.6 vol of isopropanol (~300 µl) was added and mixed gently until DNA precipitated, then centrifuged for 5 min and the supernatant was discarded. The supernatant was washed with 1 ml of 70% ethanol, mixed, centrifuged for 5 min, and the supernatant was discarded, then it was dried for 10 min at a velocity vac/45 °C. Finally it was re-suspended in 100 µl TE buffer. DNA concentration was read using 2 µl in Nanodrop machine using TE buffer as blank and the concentration was made up to 100 ng/µl in each sample.

2.3. DNA amplification by PCR

The 16S rRNA was amplified with a pair of specific primers, a 25 nucleotide forward primer: 16SF1(5′-GGAATTCAAAGG AATTGACG GGGGC-3′) and a 29-nucleotide reverse primer: 16SR2(5′-CGGGATC CCAGGCCCGGGAACGTATT-CAC-3′) [10]. The primers of 16S rRNA gave PCR products equal to (~ 479 bp). The gap gene was amplified with a pair of specific primers, a 26-nucleotide forward primer: GF1(5′-ATG GTTTTGGTAGAATTGGTCGTTTA-3′) and a 25-nucleotide reverse primer: GR2(5′-GACATTTCGTTATCA TACCAAGCTG-3′) [12]. The primers of gap gene gave a PCR product equal to (~933 bp). Finally the nuc gene was amplified with specific primers, a 21-nucleotide forward primer: NF1(5′-GCGATTGATGGTGATACGGTT-3′) and 25-nucleotide reverse primer: NR2(5′-AGCCAAGCCTTGAAC GAACTAAAGC-3′ [14]. The primer of nuc gene gave a PCR product equal to (~270 bp). The following reaction mixture was added to each sample: 2 µl DNA (100 ng), 2 µl primer (100 pmol), PCR mixture (1.5 µl $MgSO_4$, 2.5 µl 10× PCR buffer, 0.5 µl dNTPs, 0.2 µl Taq polymerase) and completed to 25 µl volume by H_2O. Initial denaturation at 95 °C for 5 min was followed by 37 cycles of amplification (denaturation at

95 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 60 s) and ending with final extension at 72 °C for 10 min. For the visualization of the product, 10 µl of the each PCR reaction was mixed with 5 µl 5× loading dye and loaded on 1.5% agarose gel for electrophoresis and visualization of the amplified PCR products. A 100 bp molecular weight DNA ladder was used for the validation of length of the amplified products (Vivantis Technologies).

3. Antibiotic Susceptibility Tests

Antibiotic susceptibility tests were performed on the all isolates of *S. aureus* by a disc diffusion method. After the broth culture, the isolates of *S. aureus* were incubated at 37 °C for 18–24 h, the fresh cultures were prepared to apply antibiotics susceptibility tests. 100 µl from each isolate suspension was cultured on LB agar or Mueller Hinton agar by cotton swab. Antibiotic Susceptibility tests of the isolates were studied against a group of ten different antibiotic discs. The plates were incubated at 37 °C for 18–24 h. Inhibition zone diameters were measured and studied using norms of the National Committee on Clinical Laboratory Standards (NCCLS).

4. Results

4.1. Bacterial identification

Isolates of *S. aureus* were cultured on LB agar and MSA mediums. Isolates showed yellow-colonies on LB agar medium (Fig 1). They fermented mannitol in MSA medium and produced yellow-colored colonies surrounded by yellow (Fig 2). *S. aureus* was identified as gram- positive cocci in grape like clusters under microscope.

4.2. DNA amplification by PCR

PCR amplification of the 16S rRNA, gap gene and nuc gene were done for all isolates to detect the species of *S. aureus*. In this study six of the specific primers were used to detect 16S rRNA region, gap gene and nuc gene. The detection and identification of the bacteria based on their 16S rRNA have many advantages. For example, each bacterial cell contains multiple copies of 16S rRNA in its ribosomes. In this study

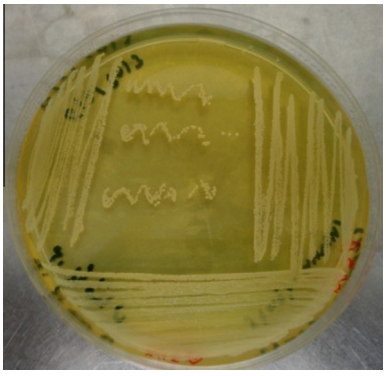

Figure 1 *S. aureus* on LB Agar.

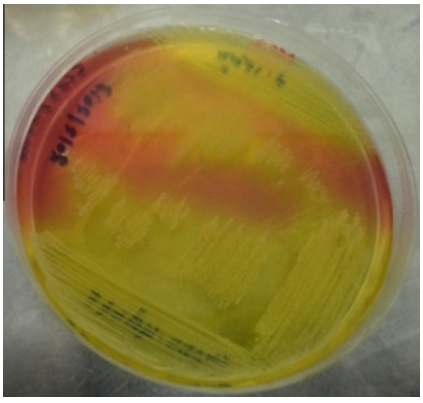

Figure 2 *S. aureus* on (MSA).

the PCR product appeared as a single band DNA with a size equal to 479 bp fragment corresponding to the 16S rRNA (Fig 3). The gap gene encoded to enzyme glyceraldehyde-3-phosphate dehydrogenase was discovered in the cell walls of *S. aureus* and other coagulase negative staphylococci. This study showed the PCR product of gap gene as a single band DNA with a size equal to 933 bp fragment (Fig 4). The nuc gene encoded to enzyme thermonuclease was used for detection of *S. aureus* causing bacteremia. Finally the PCR product appeared a single DNA band with a size equal to 270 bp fragment corresponding to the nuc gene (Fig 5). All isolates of *S. aureus* gave the corresponding PCR bands equivalent to the 16S rRNA region, gap gene and nuc gene.

4.3. Antibiotic susceptibility tests

Antibiotic susceptibility tests were evaluated by the disk diffusion method for all isolates of *S. aureus* (Fig 6). Isolates of *S. aureus* showed different level of resistance against some antibiotics. Inhibition zone diameters were measured depending on NCCLS (Fig 7). The results of Antibiotic susceptibility tests for 10 different antibiotics were as the following: Chloramphenicol (97.5%), Tetracycline (50.6%), Cefuroxime (37.0%), Levofloxacin (37.0), Erythromycin (35.8%), Ciprofloxacin (32.1%), Rifampicin 7.4%), Oxacillin (33.3%), Vancomycin (3.7%) and Imipenem (0%), (Table 1 & Fig 8).

5. Discussion

S. aureus causes a lot of problems in many hospitals worldwide and it shows increasing resistance against many of the antibiotics. In this study the results of bacterial culture for a total of 81 isolates on LB agar and MSA revealed *S. aureus* with all isolates appeared as gram-positive. Some reports indicated that several laboratories in developing countries perform screening for presumptive *S. aureus* based on growth on (MSA) and/ or DNase test [17]. In the present study *S. aureus* was identified and classified by PCR using specific primers for housekeeping genes in these bacteria, such as 16S rRNA region, gap gene and nuc gene. The PCR depending on a diagnostic protocol was used to detect the different genes [18]. It has been also found that fragments of the 16S rRNA region of all isolates were equal to 479 bp (Fig 1A). The 16S rRNA

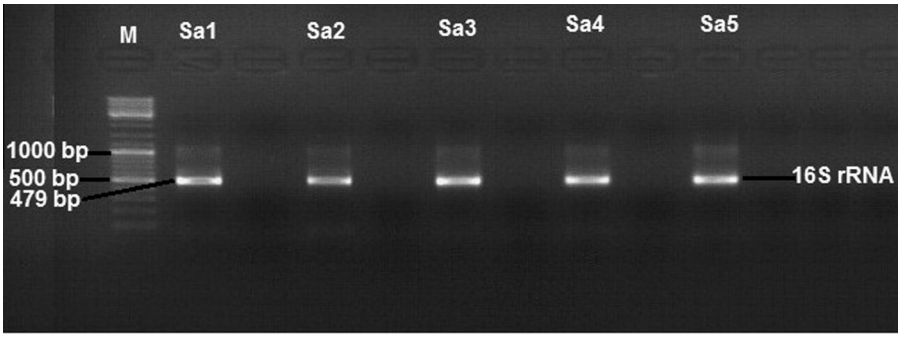

Figure 3 Gel electrophoresis shows the 16S rRNA fragments of *S. aureus*, M: DNA marker, $Sa_{(1\text{-}5)}$: *S. aureus*.

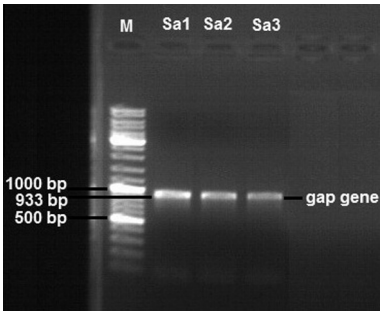

Figure 4 Gel electrophoresis shows the gap gene fragments of *S. aureus*, M: DNA marker, $Sa_{(1\text{-}3)}$: *S. aureus*.

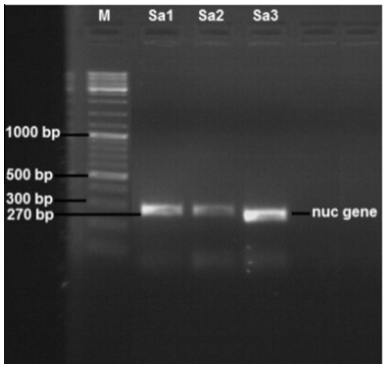

Figure 5 Gel electrophoresis shows the nuc gene fragments of *S. aureus*, M: DNA marker, $Sa_{(1\text{-}3)}$: *S. aureus*.

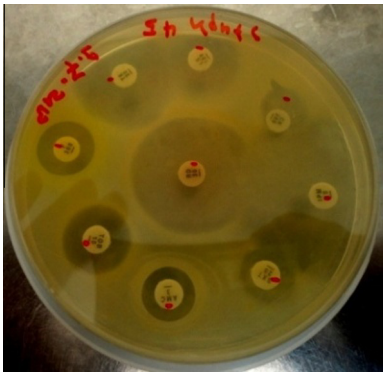

Figure 6 Disc diffusion method with inhibition zones for some antibiotics against *S. aureus*.

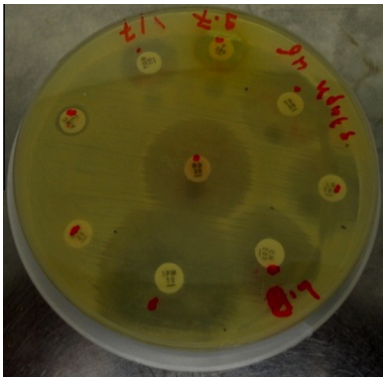

Figure 7 Disc diffusion method with Chloramphenicol-zone diameter equal to 0 mm and Vancomycin-zone diameter $\geqslant$20 mm.

and 21 protein made up the 30S subunit [19]. The 16S rRNA was used to detect isolates of *S. aureus* in patients with brain abscess and the reports indicate that the 16S rRNA fragment was equal to 479 bp [10,20]. The gap gene was used in this study to detect *Staphylococcus* sp., and the PCR product for gap gene fragment in *S. aureus* was equal to 933 bp (Fig 1B). The gap gene encodes to enzyme (glyceraldehyde-3-phosphate dehydrogenase) and analysis of the enzyme represented a high-throughput reproducible method that allows the identification of distinct *Staphylococcus* species [21]. The nuc gene is baseline in identification and classification of *S. aureus* and in the present study the nuc gene fragment was equal to 270 bp (Fig 1C). Some reports indicated that the nuc gene was encoded to enzyme thermonuclease and the length fragment of nuc gene was equal to 270 pb [22]. In our study the isolates of *S. aureus* revealed increasing resistance against many antibiotics, such as Chloramphenicol while they appeared sensitive to other antibiotic like Vancomycin. 97.5% isolates of *S. aureus* were Chloramphenicol-resistant, 50.6% were Tetracycline-resistant and 3.7% were Vancomycin-resistant. In other reports the percentage of Tetracycline-resistant *S. aureus* isolates was equal to 47.4% or 60.4% [23,24]. Most isolates of *S. aureus* were sensitive to Vancomycin and all isolates were sensitive to Rifampicin. The reports indicated that all clinical samples of *S. aureus* were resistant to Vancomycin [22,24], while the 3.85% of

Table 1 Percentage of antibiotic-resistant isolates of *S. aureus*.

Antibiotic	Number of isolates (%)		
	R	*I*	*S*
Cloramphenicol	79 (97.5)	2 (2.5)	0 (0)
Tetracycline	41 (50.6)	4 (5.0)	36 (44.4)
Cefuroxime	30 (37.0)	5 (6.2)	46 (56.8)
Levofloxacin	30 (37.0)	36 (44.4)	15 (19.0)
Erythromycin	29 (35.8)	39 (48.1)	13 (16.1)
Oxacillin	27 (33.3)	0 (0)	54 (66.7)
Ciprofloxacin	26 (32.1)	0 (0)	55 (67.9)
Rifampicin	6 (7.4)	5 (6.2)	70 (86.4)
Vancomycin	3 (3.7)	14 (17.3)	64 (79.0)
Imipenem	0 (0)	2 (2.5)	79 (97.5)

R, resistant; *I*, intermediate; *S*, sensitive.

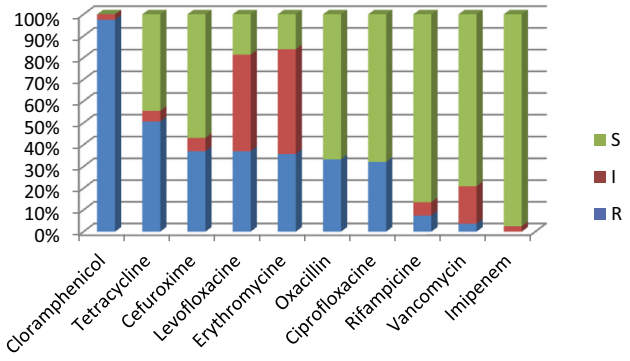

Figure 8 Scheme showing antibiotic-resistant isolates of *S. aureus* against some antibiotics, *R*, resistant; *I*, intermediate; *S*, sensitive.

clinical samples were Rifampicin-resistant and the Rifampicin was an important antibiotic in treatment *S. aureus* infection and tuberculosis [25,26]. Most of the reports suggested Vancomycin as a credible drug for treating *S. aureus* infection [27,28]. Accordingly, the results in this study proved that PCR was a specific and effective method for classifying and identifying isolates of *S. aureus* and that they showed increasing resistance against many antibiotics.

6. Conclusion

In present study the results showed that identifying and classifying *S. aureus* by PCR was a golden standard. The isolates of *S. aureus* showed increasing resistance against many antibiotics as Chloramphenicol, Levofloxacin and Erythromycin, while they were sensitive against other antibiotics, such as Vancomycin and Imipenem. Vancomycin was still the most effective drug against *S. aureus* infection as described permanently by doctors at hospitals in Damascus.

Conflict of interest

The authors declare no conflict of interest.

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